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Global-warming-caused changes of temperature and oxygen alter the proteomic profile of sea cucumber *Apostichopus japonicus*

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ABSTRACT

Multiple environmental stressors caused by global warming influence the regulation of proteins involved in various biological processes in aquatic organisms. As an important component of the marine ecosystem, sea cucumber is a suitable echinoderm species for researching stress responses. In this study, we have investigated the proteomic response of respiratory trees in the sea cucumber *Apostichopus japonicus*, challenged with environmental stresses by using the iTRAQ technique. Results showed that 262, 155 and 433 proteins were differentially regulated in response to heat, hypoxia, and heat plus hypoxia, respectively. Expression of key genes and proteins were measured by real-time PCR and western blot to validate the accuracy of the proteome files. Results showed that the interaction between these two stressors has an additive effect at the proteome level. Proteins involved in amino acid and carbohydrate metabolic processes were largely induced by heat while repressed by hypoxia. Exposure to multiple climate change stressors caused several proteins involved in lipid metabolic progress to be down-regulated and lipid catabolic processes were induced. Additionally, hypoxia and the combined stress induced proteins involved in iron homeostasis. ATP synthesis and gluconeogenesis were induced under heat and the combined stress, while ATP and glycogen synthesis were depressed under hypoxia. Proteins related with immune and defense response were largely induced and protein synthesis ability was depressed under all three stresses. Thus, sea cucumber may adopt different strategies to cope with varied environmental stress, and the situation in heat group is more similar with the combined treatment than hypoxia group. These proteomic changes in response to high-temperature and low-oxygen levels may provide insights into the defense strategies of sea cucumber in response to global climate changes.

Significance of the study: The study focused on comparative quantitative proteomics on *Apostichopus japonicus* respiratory tree using iTRAQ in responses to multiple climate change stressors: heat, hypoxia and the combined stress. Data showed that *A. japonicus* can have an acute reaction in diverse biological pathways to both individual and interacting environmental stress, including substance metabolism, signal transduction, protein synthesis, immune response and energy production. Results indicated that sea cucumber adopted different strategies to cope with varied environmental stress, and the interaction between these two stressors has an additive effect at the proteome level. These results offer insight into the molecular regulation of *A. japonicus* to multiple environmental stress and reveal possible molecular events in sea cucumber under climate changes. The understanding of adaptive variation under global climate changes in aquatic organisms could be improved.

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1. Introduction

Water temperature and dissolved oxygen (DO) are two pivotal environmental factors for marine lives. The solubility of DO decreases with increased temperature, thus heat stress is commonly accompanied with hypoxia stress [1]. Previous studies in global climate change have demonstrated that the best estimates of ocean warming in the top 100 m are about 0.6 °C (RCP2.6) to 2.0 °C (RCP8.5) by the end of the 21st century [2]. Moreover, a decreasing trend of the DO level in the ocean over the last several decades were identified [3]. As the aquatic organisms are naturally exposed to increased water temperature accompanied with decreased DO, the environmental stress (heat, hypoxia and the combined stress) could have a direct effect on aquatic organisms. Therefore, an understanding of the mechanisms adopted by marine organisms in response to global climate change is urgently needed.

The sea cucumber *Apostichopus japonicus*, is a commercial holothurian with high nutritive and medicinal value. It is mainly distributed along the northwest coast of the Pacific Ocean from 35° to 44°N, including Russia, China, Japan and Korea [4,5]. It is an important fishery in these country with long history, and in 2015, the aquaculture area and yields in China had reached 216,508 ha and 205,791 tons, respectively [6]. In its key habitats, for example, northern Yellow sea and Bohai sea, the sea cucumber experienced the highest temperature between 26 and 30 °C. In summer, the DO level in these habitats may decrease to below 2 mg/L, which is commonly defined as hypoxia [7,8]. These are far beyond the optimal condition for the growth and survival of sea cucumbers, thus leading to high mortality rates [9,10]. In addition, echinoderms is relatively sensitive to environmental stress compared with other marine species such as molluscs, ascidians, and anthozoans [11]. Moreover, sea cucumber, as a deposit feeder, is an important component of the marine ecosystem and could play a significant role in carbon cycling as fixed carbon can be redistributed through its harvest. The special characteristics of sea cucumber suggest that it is not only a significant aquaculture species but also a suitable organism for studying the molecular mechanisms of marine organisms under multiple climate changed stressors.

Temperature is an important environmental factor, and it was proved to negatively affect sea cucumber in various aspects, including growth [4,12–14], body weight [15], feeding behavior [4,14], gut mass [16], fertilization ability of gametes [17], and would change immune capacities [18,19], activities of enzymes [12,18,19], oxygen consumption rates [13,20], metabolism [21], fatty acid profiles [22] and HSP70 expression [12,15,18]. Exposure to low DO also results in changes to *A. japonicus*, including non-specific immunity, oxidative stress responses, oxygen consumption rate, metabolic enzymes activities, metabolites concentration and expression of miRNAs [23–27]. However, to the best of our knowledge, the molecular mechanisms of sea cucumber under environmental stress have not been fully investigated, especially at the proteomic level and under multiple stressors.

Proteomic analysis, which provides more reliable quantitative measurements and comparisons of samples, has been a powerful tool for investigating cellular responses to environmental factors and one the most well-known protein quantitative technologies is isobaric tags for relative and absolute quantification (iTRAQ) [10,28–30]. In the research field of responses of aquatic organisms under environmental stress, fish, curstaceans and mollusca are commonly recognized as popular species. For example, proteomics responses of sturgeon (*Acipenser medirostris* and *Acipenser transmontanus*) [31], clam (*Mercenaria mercenaria*) [32], Pacific oyster (*Crassostrea gigas*) [33,34], blue mussel (*Mytilus galloprovincialis* and *M. trossulus*) [35,36]) and sea squirts (*Cinona intestinalis* and *C. savignyi*) [37] to heat stress and Darkbarbel catfish (*Pelteobagrus vachelli*) [38], zebrafish (*Danio rerio*) [39], crucian carp (*Carassius carassius*) [40], Chinese shrimp (*Fenneropenaeus chinensis*) [41], oriental river prawn (*Macrobrachium nipponense*) [42] and great scallop (*Pecten maximus*) [43] to hypoxic stress. Proteomic studies in sea cucumber have been reported from several aspects, including intestine regeneration [30], albino and purple strains [44,45] and impacts of single environmental factor, such as heat shock [10,46,47] and pathogen challenge [48]. In conclusion, the group of echinoderms is certainly underrated in proteomics studies, especially about the stress response. The effects of heat, hypoxia and heat plus hypoxia on sea cucumber remain unclear, in particular, the responses under combined stressors. Hence, the effect of multiple climate stressors on the proteome changes in sea cucumber urgently need to be uncovered.

In this study, the proteomic profile of sea cucumber was conducted to identify key proteins involved in stress resistance based on the iTRAQ technique. The respiratory tree is the major tissue responsible for the respiration and metabolism of sea cucumbers under adverse conditions [26]. Thus, for this study, it was chosen as the source tissue. Real-time PCR (RT-qPCR) and western blot were used to validate the accuracy of the proteomic data. The changes of processes like metabolism, signal transduction, protein biosynthesis, immune defense and energy production were illustrated. These results may offer insight into the molecular regulation of *A. japonicus* to high temperatures and hypoxic environments and reveal possible molecular events of sea cucumber in warming and lower-oxygen oceans, thus increasing the understanding about the potential adaptive mechanism of marine organisms under global climate change.

2. Material and methods

2.1. Animals

Sea cucumbers (*A. japonicus*) were collected from the coast of Weihai, China, the wet weights were 90–110 g. After weighing, they were acclimated in tanks containing aerated sand-filtered seawater at 16 ± 0.5 °C for one week before use and were fed once a day at 8:00 am. Remaining feed was removed daily during the acclimation and experimental periods. Healthy sea cucumbers were randomly selected from acclimation tanks and divided into groups. One group was maintained as the normal control (NC) in water with sufficient dissolved oxygen (DO 8 mg/L) and a suitable temperature of 16 °C. The hypoxia treatment group (LO) was maintained in water with low dissolved oxygen (DO 2 mg/L) and a suitable temperature of 16 °C. The heat stress treatment group (HT) was maintained at a high temperature of 26 °C and sufficient dissolved oxygen. The heat and hypoxia combined treatment group (HL) was maintained with a high temperature of 26 °C and low dissolved oxygen concentration (DO 2 mg/L). Acclimation was carried out by gradually increasing the ambient temperature from 16 °C to 26 °C at the speed around 2 °C/h and decreasing the ambient dissolved oxygen level from 8 mg/L to 2 mg/L at the speed around 1 mg/L/h using a 2-kW heating rod and dissolved oxygen control system, respectively [10,49]. The moment when the water temperature rose to 26 °C and/or the dissolved oxygen decreased to 2 mg/L was regarded as the initial time. All the sea cucumbers from each group were cultivated in separated tanks according to the biological replicates set and exposed to stress for 48 h. Nine sea cucumber individuals (three biological replicates × three sea cucumbers per biological replicate) for each experiment were dissected promptly from each group and respiratory trees were sampled, preserved in liquid nitrogen, and stored at −80 °C. Ethics Statement is not applicable. Human and vertebrate species or samples were not involved in our research. No permission was needed for sea cucumber collection. The sea cucumber (*A. japonicus*) is not an endangered or protected species.

2.2. Protein extraction, digestion, and iTRAQ labeling

Frozen respiratory tree tissues were prepared for the iTRAQ analysis. The tissue was ground to powder in liquid nitrogen and dissolved in lysis buffer 3 (7 M urea, 2 M thiourea, 4% CHAPS, and 40 mM Tris-HCl, pH 8.5) containing 1 mM PMSF and 2 mM EDTA. After 5 min,

dithiothreitol (DTT) was added to a final concentration of 10 mM. An ultrasound was carried out on ice for 10 min to mix the suspension, which was then centrifuged at 25,000 g for 20 min at 4 °C. The supernatant was extracted and incubated at 56 °C for 1 h and alkylated immediately with 55 mM iodoacetamide in the dark at room temperature (~ 25 °C) for 45 min. The treated proteins were precipitated in four times the volume of acetone at −20 °C for 2 h. The supernatant containing proteins was quantified using the Bradford method following centrifugation at 25,000 g for 20 min at 4 °C.

The protein solution (100 μg) containing 8 M urea was diluted four times with 100 mM TEAB. Trypsin gold (Promega, Madison, WI, USA) was used to digest the proteins at a 40:1 ratio of protein: trypsin at 37 °C overnight. Following trypsin digestion, peptides were desalted using a Strata X C18 column (Phenomenex, Torrance, CA, USA) and vacuum dried. Peptides were dissolved in 30 μL 0.5 M TEAB with vortexing. After iTRAQ labeling, reagents were recovered at ambient temperature, then transferred and combined with proper samples. Peptide labeling was performed on normal control conditions (NC: 119 Da), samples under high temperature (HT: 117 Da), samples under low DO (LO: 121 Da) and samples under high temperature and low DO (HL: 118 Da) using four different labels from an iTRAQ reagents 8-plex applications kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The labeled peptides were desalted using a Strata X C18 column (Phenomenex) and vacuum dried.

2.3. Peptide fractionation and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis

The labeled samples were fractionated on a SCX column using a LC-20AB high performance liquid chromatography (HPLC) pump system (Shimadzu, Kyoto, Japan). Peptides were reconstituted using 2 mL buffer A [5% acetonitrile (ACN) and 95% H_2O (pH 9.8)] and loaded onto a column containing 5-μm particles (Phenomenex). Peptides were separated at a flow rate of 1 mL/min using a gradient of 5% buffer B [5% H_2O and 95% ACN (pH 9.8)] for 10 min, 5% to 35% buffer B for 40 min, and 35% to 95% buffer B for 1 min. The system was maintained in 95% buffer B for 3 min, followed by a decrease to 5% within 1 min prior to equilibration with 5% buffer B for 10 min. Elution was monitored by measuring absorbance at 214 nm, and fractions were collected every 1 min. Eluted peptides were pooled into 20 fractions and vacuum dried. Each fraction was resuspended in buffer A (2% ACN and 0.1% FA in water) and centrifuged at 20,000 g for 10 min. Peptides were eluted from the trap column and separated using a LC-20AD nano-HPLC instrument (Shimadzu) with an analytical C18 column (inner diameter: 75 μm, length: 15 cm) packed in house. The gradient was initiated at 300 nL/min starting from 8% to 35% buffer B (2% H_2O and 0.1% FA in ACN) for 35 min, followed by an increase to 60% over 5 min, then maintenance in 80% buffer B for 5 min before returning to 5% buffer B and equilibrating for 10 min. Data were acquired using a TripleTOF 5600 system (SCIEX, Framingham, MA, USA) equipped with a Nanospray III source (SCIEX), a pulled quartz tip as the emitter (New Objectives, Woburn, MA, USA), and controlled with the software Analyst 1.6 (AB SCIEX, Concord, ON, Canada). Data were acquired with the following MS conditions: ion spray voltage, 2300 V; curtain gas, 30; nebulizer gas, 15; and interface-heater temperature, 150 °C. The high-sensitivity mode was used for data acquisition, with an accumulation time for MS1 at 250 ms and mass ranges from 350 Da to 1500 Da. Based on the intensities observed during the MS1 survey, as many as 30 product-ion scans were collected when exceeding a threshold of 150 counts/s, and with a charge state of 2^+ to 5^+, dynamic exclusion was set for 1/2 of the peak width (12 s). For iTRAQ data acquisition, the collision energy was adjusted to all precursor ions for collision-induced dissociation and the Q2 transmission window for 100 Da was 100%.

2.4. Protein identification and quantification

The raw LC-MS/MS data were converted to MGF files using Proteome Discovery 1.2 (Thermo, Pittsburgh, PA, USA). The proteins were identified using Mascot search engine 2.3.02 (Matrix Science, London, UK) with the *A. japonicus* database from previous studies [50–52]. For protein identification, the following search parameters were selected, Enzyme: Trypsin, Mass Values: Monoisotopic, Fixed modification: Carbamidomethyl (C), iTRAQ 8plex (N-term), iTRAQ 8plex (K), Variable modification: Oxidation (M), iTRAQ 8plex (Y), Peptide mass tolerance: 0.05 Da, Fragment Mass Tolerance: 0.1 Da. All identified peptides had an ion score above the Mascot peptide identity threshold, and a protein was considered identified if at least one such unique peptide match was apparent for the protein. The IQuant software (BGI, Shenzhen, China) was used for quantitatively analyzing the peptides labeled with isobaric tags [53]. During the process of quantification, variance stabilization normalization (VSN) was used for normalization; the weighted averages were used for calculation of protein ratios Permutation tests were used for next statistical analysis. All the proteins with a false discovery rate (FDR) < 1% will proceed with downstream analysis including GO, COG and KEGG Pathway. Proteins with p-values < .05 and fold changes (FC) > 1.20 or < 0.83 were identified as being differentially regulated [54].

2.5. Real-time polymerase chain reaction (PCR) and western blot validation

Real-time PCR was performed in order to illustrate the expression of important genes encoding differentially regulated proteins. Total RNA was extracted from the respiratory tree, which is the same tissue used for the construction of the proteomic profile, by using MiniBEST Universal RNA Extraction Kit (Takara, Shiga, Japan) following the manufacturer's instructions. The quality and concentration of RNA were measured by NanoDrop 1000 (Thermo Fisher Scientific, USA), and three high-quality RNA samples extracted from three different sea cucumbers in each group were selected for further experiments. First-strand cDNA was synthesized as the template using reverse transcriptase (Takara). The mRNA expression levels were examined using a SYBR Green® real-time PCR assay (SYBR PrimeScrip ™ RT-PCR Kit II, Takara) with an Eppendorf Mastercycler® ep realplex (Eppendorf, Hamburg, Germany), and β-actin was used as a reference gene for internal standardization [55]. Primers (Table S1) were designed for optimal performance with primer3 according to the sequence information in the transcriptome database. The total volume of the amplification mix was 20 μL, which contains 8 μL of RNase-free water, 10 μL of SYBR GreenMasterMix (Takara), 1 μL of diluted cDNA, 0.5 μL (each) of forward and reverse primer (10 mM). The thermal cycling was performed according to the following procedure: 95 °C for 5 s, followed by 40 cycles at 95 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s. The specificity of the amplification products was illustrated by melting curve analysis. The $2^{-\triangle\triangle CT}$ method was used to analyze the comparative mRNA expression levels of the selected genes [56]. The analysis was performed by SPSS19 software (IBM Corp., Armonk, NY, USA). All the data were indicated as mean ± standard deviation (SD). Significance analysis between different groups of each gene was analyzed by one-way ANOVA with Tukey's test, and the threshold of statistical significance was $P < .05$.

The expression profiles of key responsive proteins involved in heat and hypoxia stress were further validated using western blot analysis, such as heat shock protein 10 (HSP10), heat shock protein 70 (HSP70), hypoxia-inducible factor-1a (HIF-1a) and factor inhibiting HIF-1 (FIH-1). β-actin was selected as the internal reference. Respiratory trees had three biological replicates in each group from the backup materials for iTRAQ, which were extracted using a total protein extraction kit (C510003-0050, Sangon Biotech, Shanghai, China). Total proteins were then separated by 10% SDS polyacrylamide gel. After electrophoresis, the proteins were electro transferred to PVDF membrane (F619537-

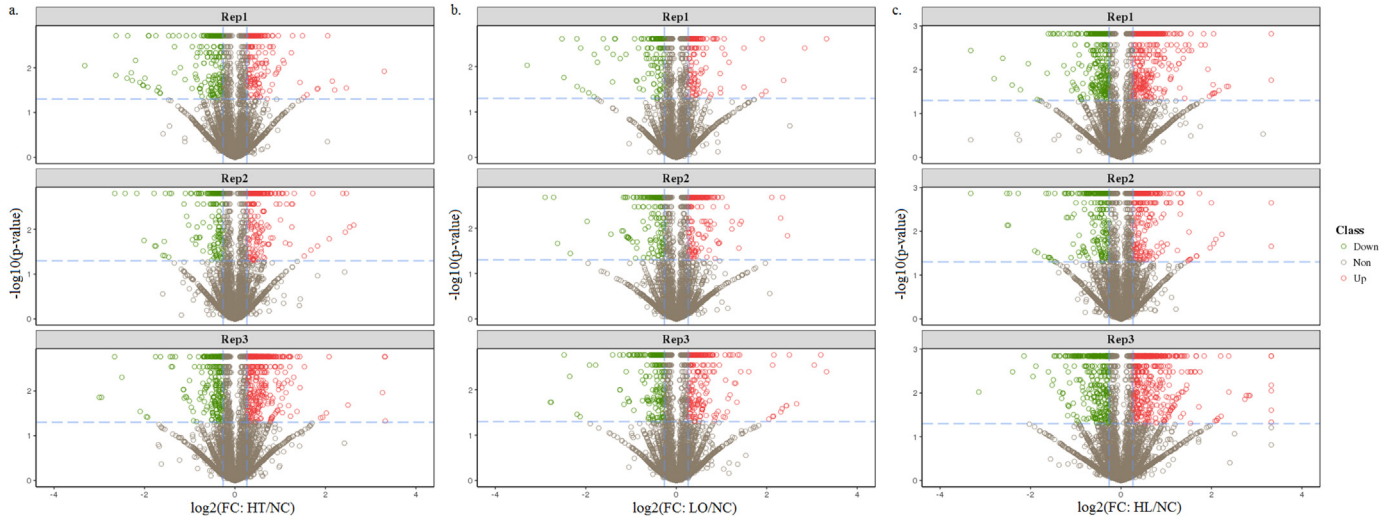

Fig. 1. The change level of global proteins in sea cucumber in the treatment groups compared with normal control group (a. HL vs NC; b. HT vs NC; c. LO vs NC). Only the proteins with $\log_2$Ratio (treatment group/normal control group) > 0.26 or < −0.26 were colored (FC > 1.20 as red and < 0.83 as green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

0001; Ewell, Guangzhou, China). After blocking for 1 h in TBST (0.05% Tween 20, 150 mM NaCl, pH 7.5, 50 mM Tris/HCl) with 5% albumin bovine V (Solarbio, Beijing, China) at room temperature, membranes were incubated with primary antibody, including β-actin (1:800), HSP10 (1:500), HSP70 (1:800), HIF-1a (1:1000) and FIH-1 (1:800) against rabbit (D161766; D220786; D162108; D162109; Sangon) for overnight. After three washes with TBST, the membranes were incubated with goat anti-rabbit IgG secondary antibody (1:7000; D110058-0100, Sangon) for 1 h. After six washes with TBST, the membranes were visualized using chemiluminescence reagent (C506668-0100, Sangon). Densitometry analysis was performed using ImageJ software.

2.6. COG, GO and KEGG pathway enrichment analyses

The Clusters of Orthologous Groups database (COG: http://www.geneontology.org/), Gene Ontology (GO: http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.genome.jp/kegg/) databases were used to classify and group the identified proteins. The hypergeometric test was used to identify significantly enriched COG categories, GO terms and pathways of differentially regulated proteins. COG, GO and KEGG pathway enrichment analyses were based on significantly up-regulated proteins and down-regulated proteins, respectively ($P < .05$). The rich factor is the ratio of differentially regulated protein number annotated in a pathway term to all protein numbers annotated in the same pathway term. A greater rich factor means a greater intensiveness. A p-value < .05 was considered as significant. All unique and shared differentially regulated proteins in the three groups were presented in a Venn diagram using Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

3. Results

3.1. Overview of the proteomics data

In the current study, protein-expression profiles of sea cucumbers respiratory tree under different climate changed stressors were constructed. All raw proteomic data were submitted to the ProteomeXchange (Database ID: PXD008472). A total of 980,646 spectra were obtained, of which 171,103 unique spectra were detected. A total of 8437 proteins and 36,397 peptides were identified at a global false discovery rate (FDR) of 1% (Table S2). The global regulation changes of these proteins under heat, hypoxia and the combined stress (heat plus hypoxia) are shown in Fig. 1. In the present study, significantly regulated proteins were identified by specific criteria (p-values < .05 and FC > 1.20 or < 0.83). Compared with normal control conditions (NC), 262 proteins showed significant responses to high-temperature treatment (heat stress, HT), with 152 up-regulated proteins and 110 down-regulated proteins; 155 proteins showed significant responses to low dissolved oxygen treatment (hypoxia stress, LO), with 80 up-regulated proteins and 75 down-regulated proteins; 433 proteins showed significant responses to high temperature and low dissolved oxygen treatment (heat plus hypoxia stress, HL), with 239 up-regulated proteins and 194 down-regulated proteins. A Venn diagram shows the specific and common number of differentially regulated proteins in the three groups (Fig. 2). Among these, 22 were classified as common proteins, 86 as specific proteins in the HT group, 102 as specific proteins in the LO group and 254 as specific proteins in the HL group.

Representative up-regulated proteins (FC ≥ 1.50, P < .05) and down-regulated proteins (FC ≤ 0.75, P < 0.05) in sea cucumber under heat, hypoxia and heat plus hypoxia stress compared with the control group are shown in Table 1. According to the fold change in the comparison of HT and NC group, five of the most significant up-regulated proteins were Titin, Solute carrier family 22 member 6, Semaphorin-1A, Lysozyme 3 and Prominin-1-A; and five of the most significant down-regulated proteins under heat stress were Serine/threonine-protein phosphatase 4 regulatory subunit 4, Small subunit processome component 20 homolog, Tryptophan 2,3-dioxygenase (TDO2), Acid phosphatase type 7 and Tryptophan-tRNA ligase, cytoplasmic (WARS). According to the fold change in the comparison of LO and NC group, five of the most significant up-regulated proteins were fatty acid-binding protein, uncharacterized protein LOC593133, prominin-1-like and Tenascin-R; and five of the most significant downregulated proteins under hypoxia stress were Small subunit processome component 20 homolog, Immune-associated nucleotide-binding protein 7, WARS, TDO2 and Acyl-CoA-binding protein. According to the fold change in the comparison of HL and NC group, five of the most significant up-regulated proteins were Putative methyltransferase DDB_G0268948, Titin, uncharacterized protein LOC100888387 isoform 1, Luminal-binding protein 1 and Luminal-binding protein 5; and five of the most significant downregulated proteins under heat plus hypoxia stress were WARS, Carbohydrate sulfotransferase 15, Acid phosphatase type 7, Contactin-5 and Splicing factor, proline- and glutamine-rich. Detailed information about significantly regulated proteins could be found in Table S3.

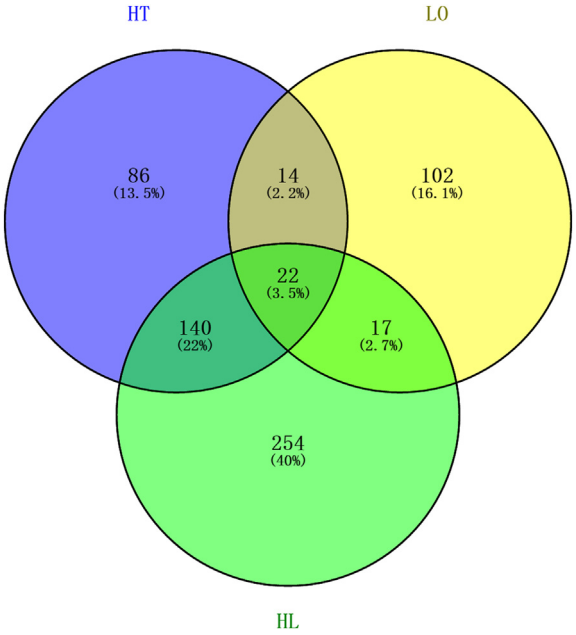

Fig. 2. Venn diagram of unique and shared differentially regulated proteins among environmental stresses.

3.2. Characterization of mRNA- and protein-level expression

A total of 12 encoding genes of differentially regulated proteins were selected for a further verification experiment, including *WARS, TDO2*, *Dystonin (DST), heat shock protein 10 (HSP10), heat shock protein 70 (HSP70), Glutathione S-transferase (GSTP1), Neogenin (NEO1), Alcohol dehydrogenase [NADP(+)] A (ADH), Thioredoxin (TXN), Fibrinogen C domain-containing protein 1 (FIBCD1), Ubiquitin carboxyl-terminal hydrolase isozyme L3 (UCHL3),* and *angiotensin-converting enzyme (ACE)*. Moreover, *WARS*, *TDO2*, *DST*, *GSTP1*, *NEO1*, *ADH, TXN* and *UCHL3* were significantly decreased and *HSP70* was significantly increased under environmental stress compared with normal control conditions. *HSP10* was significantly increased under heat and heat plus hypoxia stress compared with normal control conditions. However, proteomics data showed that ACE was significantly up-regulated in sea cucumber under three treatments, but *ACE* gene was only significantly up-regulated under heat plus hypoxia stress. *FIBD1* showed down-regulated under environment stress at transcriptional level, which is different with the pattern at proteomics level in the present study. The ten of twelve encoding genes (*HSP10, HSP70, WARS*, *TDO2*, *DST*, *GSTP1*, *NEO1*, *ADH, TXN* and *UCHL3*) share similar expression tendencies with those from the proteomic data, indicating that our proteomic data are reproducible and solid (Fig. 3). Furthermore, we performed western blotting analysis on some selected proteins related with heat response (HSP10 and HSP70) and hypoxia response (HIF-1a and FIH-1) (Fig. 4). Regardless of the significance level, HIF-1a showed upregulated and FIH-1 showed downregulated tendency in sea cucumber under hypoxia stress. The tendency is same as the general results in organisms exposed to low dissolved oxygen. Moreover, HSP10 was significantly increased in sea cucumber under heat stress compared with heat plus hypoxia stress. Some proteins expression levels were not significantly changed, like HSP70, and that might because of the delayed effect of transcription from genes to proteins. The RT-qPCR and western blot analysis revealed that these genes and proteins share similar expression tendencies with those from proteomic data, suggesting that the proteomic data are reproducible and reliable).

3.3. Functions of differentially regulated proteins under environmental stress

The functional analyses of all the identified differentially regulated proteins under environmental stress were based on Cluster of Orthologous Groups of proteins (COG) and were summarized into following 11 categories: energy production and conversion, cell growth and division, signal transduction mechanisms, general function, biosynthesis and modification, amino acid metabolism, carbohydrate metabolism, lipid metabolism, small molecule metabolism, secondary metabolites metabolism and others/function unknown (Fig. 5).

In general, the number of differentially regulated proteins in the HL group was more than the HT and LO group in all 11 categories, indicating that the interaction between these two stressors has an additive effect at the proteome level. The results showed that the HT group accounts for a larger percentage in biosynthesis and modification (34.4%), amino acid metabolism (5.1%) and small molecule metabolism (3.5%) compared with the LO group. The LO group accounts for a larger percentage in signal transduction mechanisms (5.7%), cell growth and division (5.0%), lipid metabolism (1. 9%) and secondary metabolites metabolism (3.1%) compared with the HT group. Moreover, the HL group accounts for a larger percentage of lipid metabolism (2.4%) compared with the HT and LO group. These results suggested that stress resistance is a complex and various biological process.

3.4. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment analyses

3.4.1. GO enrichment analyses of up- and down-regulated proteins in sea cucumber under environmental stress

Enriched GO terms based on significantly up- and down-regulated proteins in sea cucumber under heat, hypoxia and the combined stress were analyzed in this study ($P < .05$), and the top five enriched GO terms by cluster frequency in “cellular component” (CC), “molecular function” (MF) and “biological process” (BP) are shown in Table S4. The number of significantly enriched categories based on differentially regulated proteins in sea cucumbers under environmental stress is shown in Table S5.

From the results of BP categories, we found that the results were similar in HT and HL group. For example, based on the significantly changed proteins in HT and HL group compared with NC group enriched process, “translation”, “translational elongation”, “macromolecule biosynthetic process” and “cellular macromolecule biosynthetic process” were upregulated; “RNA processing”, “mRNA Processing” and “RNA splicing” were downregulated. However, things were quite different in LO group. Iron ion related biological processes were significantly enriched based on the upregulated proteins in LO group compared with NC group, such as “iron ion transport”, “cellular iron ion homeostasis” and “iron ion homeostasis”. Moreover, based on the downregulated proteins in LO group compared with NC group, “positive regulation of growth” was the most commonly represented, followed by “generation of precursor metabolites and energy” and “positive regulation of transporter activity”.

From the results of MF categories based on the upregulated proteins in treatment group compared with normal control, “structural constituent of ribosome” and “structural molecule activity” were significantly enriched in HT and HL group. Binding-related GO terms were significantly enriched in HT group, such as “RNA binding”, “rRNA binding” and “unfolded protein binding”; Iron-related GO terms were significantly enriched in LO group, such as “ferric iron binding” and “iron ion binding”. Based on the downregulated proteins in treatment group compared with normal control, helicase-activity-related GO terms were significantly enriched in HT group, including “ATP-dependent helicase activity”, “purine NTP-dependent helicase activity” and “helicase activity”; Compound-binding-related GO terms were

Table 1
Representative significantly changed proteins under environmental stress in the respiratory tree of the sea cucumber *A. japonicas*. (FC ≥ 1.5 and FC ≤ 0.75, $P < .05$).

		HT_117/NC_119		LO_121/NC_119		HL_118/NC_119	
No.	Protein description	Mean ratio	SD	Mean ratio	SD	Mean ratio	SD
Substance biosynthesis, transport and metabolism							
Amino acid transport and metabolism							
1347	Angiotensin-converting enzyme 2	1.88	0.645	1.56	0.272	2.73	1.929
6610	Betaine–homocysteine *S*-methyltransferase 1	1.59	0.327	0.96	0.153	0.84	0.108
6627	4-hydroxyphenylpyruvate dioxygenase	1.62	0.36	1.16	0.232	1.64	0.598
7830	Angiotensin-converting enzyme	1.53	0.322	1.59	0.359	1.91	0.324
8040	Tryptophan 2,3-dioxygenase	0.39	0.129	0.49	0.115	0.56	0.086
8372	*S*-methylmethionine–homocysteine *S*-methyltransferase BHMT2	1.75	0.481	1.56	0.421	1.54	0.709
Carbohydrate transport and metabolism							
1060	Sialidase-1	0.71	0.063	1.07	0.025	0.52	0.049
4395	Aldose 1-epimerase	1.29	0.278	0.97	0.073	1.54	0.298
6118	Alpha-amylase	1.37	0.556	1.31	0.133	1.62	0.405
6242	Xylose isomerase	0.73	0.097	0.87	0.055	0.77	0.077
6355	Mannose-6-phosphate isomerase	1.3	0.258	1	0.091	1.58	0.677
Lipid transport and metabolism							
5	15-hydroxyprostaglandin dehydrogenase [NAD(+)]	1.29	0.222	0.96	0.2	1.55	0.281
559	Acyl-CoA-binding protein	0.83	0.282	0.62	0.058	0.97	0.241
4455	Enoyl-CoA hydratase, mitochondrial	1.22	0.044	1.01	0.025	1.56	0.171
7836	Long-chain-fatty-acid–CoA ligase 1	0.8	0.089	0.73	0.143	0.82	0.11
Coenzyme transport and metabolism							
1917	Renilla-luciferin 2-monooxygenase	1.52	0.333	1.23	0.252	1.27	0.389
2727	Glycine *N*-methyltransferase	2.02	0.26	0.99	0.214	1.14	0.22
4782	Probable S-adenosylmethionine-dependent methyltransferase CRG1	0.74	0.033	0.84	0.23	0.94	0.145
5887	Phosphomannomutase	1.4	0.419	1.22	0.302	1.74	0.39
6383	Adenosylhomocysteinase	2.27	1.31	1.12	0.326	1.58	0.553
6513	Putative methyltransferase DDB_G0268948	3.41	2.711	2.02	2.144	6.95	5.008
Energy production and conversion							
5835	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	1.29	0.193	1.11	0.078	1.57	0.415
6289	C-terminal-binding protein	0.77	0.083	0.95	0.132	0.62	0.154
7445	Chromate reductase	0.88	0.211	0.69	0.141	1.12	0.086
Secondary metabolites biosynthesis, transport and catabolism							
106	Cytochrome P450 26B1	1.35	0.197	1.23	0.323	1.79	0.084
4022	Alcohol dehydrogenase [NADP(+)] A	1.51	0.315	1.14	0.152	1.79	0.883
4325	Aldose reductase	1.18	0.265	0.8	0.141	1.7	0.558
4635	Beta,beta-carotene 15,15′-dioxygenase	1.12	0.04	0.88	0.118	0.68	0.188
4768	Fumarylacetoacetate hydrolase domain-containing protein 2	1.15	0.251	0.85	0.075	1.77	0.511
6078	Prostaglandin reductase 1	1.6	0.457	1.38	0.327	1.64	0.458
6813	Alcohol dehydrogenase [NADP(+)] A	1.04	0.213	0.66	0.061	1.29	0.286
8213	Steroid 17-alpha-hydroxylase/17,20 lyase	0.73	0.189	0.92	0.128	0.8	0.273
Synthesis and modification							
Transcription							
228	Chromodomain-helicase-DNA-binding protein 5	0.75	0.081	1	0.041	0.54	0.1
5180	Putative transcription elongation factor spt6	0.84	0.088	1.05	0.123	0.71	0.003
Translation, ribosomal structure and biogenesis							
30	60S ribosomal protein L19	1.73	0.449	1.15	0.188	2.06	0.284
639	RNA-binding protein 8A	0.87	0.017	0.94	0.088	0.74	0.088
974	Cold-inducible RNA-binding protein	1.4	0.283	0.97	0.08	1.75	0.498
1161	ELAV-like protein 2	0.75	0.041	0.79	0.066	0.69	0.063
1703	60S ribosomal protein L31	1.57	0.34	1.17	0.206	1.68	0.218
2220	60S ribosomal protein L34-B	1.58	0.34	1.27	0.247	1.55	0.182
2792	Splicing factor, proline- and glutamine-rich	0.7	0.051	1.04	0.12	0.49	0.1
2891	60S ribosomal protein L27a	1.72	0.509	1.07	0.23	1.76	0.244
3061	Tryptophan–tRNA ligase, cytoplasmic	0.49	0.182	0.46	0.099	0.35	0.044
3117	H/ACA ribonucleoprotein complex subunit 4	0.93	0.124	1.1	0.037	0.74	0.093
3544	Elongation factor 1-alpha	1.42	0.202	1.12	0.175	1.63	0.287
3960	Poly(U)-binding-splicing factor PUF60	0.85	0.064	0.97	0.088	0.72	0.039
4162	Cleavage stimulation factor subunit 2	0.88	0.093	1.05	0.16	0.7	0.106
5879	40S ribosomal protein S20	1.47	0.232	1.13	0.188	1.54	0.045
5886	Serine/arginine-rich splicing factor 4	0.8	0.07	1.08	0.108	0.7	0.155
5929	60S ribosomal protein L26	1.56	0.345	1.13	0.163	2.13	0.133
6022	Ribonuclease Oy	0.74	0.079	0.8	0.098	0.72	0.213
6598	Eukaryotic translation initiation factor 5A-1	1.38	0.224	1.07	0.31	1.63	0.108
6667	60S ribosomal protein L35	1.49	0.262	1.18	0.259	1.82	0.249
6671	40S ribosomal protein S15	1.59	0.497	1.05	0.215	1.63	0.256
6906	Probable 28S rRNA (cytosine-C(5))-methyltransferase	0.79	0.079	1.12	0.073	0.67	0.073
7066	60S ribosomal protein L3	2.03	0.802	0.99	0.301	2.03	0.366
7613	40S ribosomal protein S23	1.4	0.192	1.05	0.272	1.76	0.212

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Table 1 (*continued*)

		HT_117/NC_119		LO_121/NC_119		HL_118/NC_119	
No.	Protein description	Mean ratio	SD	Mean ratio	SD	Mean ratio	SD
7900	Ribonuclease T2	0.92	0.327	0.7	0.08	0.75	0.258
7925	60S ribosomal protein L10a	1.61	0.401	1.02	0.207	1.79	0.175
8291	Splicing factor 3B subunit 6	0.76	0.036	1.1	0.128	0.64	0.132
Posttranslational modification, protein turnover, chaperones							
2	Predicted protein (Fragment)	0.63	0.01	0.7	0.13	0.75	0.248
60	Heat shock protein 10	1.34	0.161	0.83	0.073	1.54	0.131
1266	Collagenase 3	0.89	0.109	1.22	0.192	1.88	0.422
1316	Luminal-binding protein 1 (Fragment)	1.42	0.057	1.93	1.168	3.78	2.709
1561	Glutamate carboxypeptidase 2	1	0.61	0.91	0.158	0.55	0.122
1609	Dipeptidyl peptidase 1	0.84	0.078	1.07	0.083	0.72	0.022
2953	Heat shock protein 70	1.92	0.549	0.99	0.075	2.76	0.384
4170	Luminal-binding protein 5	1.65	0.394	1.55	0.602	3.32	1.682
5637	Glutathione S-transferase	0.87	0.152	0.79	0.194	0.58	0.139
5759	Peptidyl-prolyl cis-trans isomerase pin1	1.41	0.179	1.13	0.161	2.39	0.384
6189	Heat shock protein HSP 90-alpha	1.32	0.438	1.11	0.138	1.83	0.409
6230	Heat shock 70 kDa protein C	1.25	0.404	1.6	0.632	2.44	1.111
6271	Peroxiredoxin-5 (Fragment)	1.29	0.109	0.98	0.035	1.58	0.09
7725	Heat shock 70 kDa protein	1	0.103	1.41	0.327	1.57	0.476
8044	cathepsin *Z*-like isoform X1	0.81	0.13	0.93	0.072	0.67	0.045
Replication, recombination and repair							
1391	DNA replication licensing factor mcm7-B	0.87	0.092	0.9	0.075	0.61	0.117
1636	Spliceosome RNA helicase DDX39B	0.82	0.074	1.01	0.08	0.65	0.057
3011	Probable ATP-dependent RNA helicase DDX23	0.77	0.042	1.05	0.04	0.73	0.099
3791	ATP-dependent RNA helicase DDX18	0.77	0.043	0.95	0.105	0.66	0.093
4023	Heme-binding protein 2	0.76	0.111	0.89	0.094	0.55	0.151
4029	Intron-binding protein aquarius	0.88	0.106	0.96	0.086	0.7	0.038
5097	probable ATP-dependent RNA helicase DDX5 isoform X2	0.76	0.069	1.05	0.138	0.66	0.048
5143	DNA replication licensing factor mcm2	0.96	0.046	0.92	0.171	0.74	0.023
5392	Flap endonuclease 1	0.81	0.021	0.9	0.112	0.72	0.052
5995	U5 small nuclear ribonucleoprotein 200 kDa helicase	0.83	0.044	0.97	0.034	0.71	0.044
7660	Probable ATP-dependent RNA helicase DDX5	0.7	0.096	1.03	0.107	0.64	0.019
Cell, nuclear and chromatin pattern							
Cell wall/membrane/envelope biogenesis							
1895	Glutamine–fructose-6-phosphate aminotransferase [isomerizing] 1	1.29	0.079	1.19	0.115	1.7	0.259
3112	Tetraketide alpha-pyrone reductase 1	1.47	0.337	1.52	0.444	1.63	0.239
3664	Tropomyosin A (Fragment)	0.96	0.185	0.63	0.181	0.93	0.103
3768	Tropomyosin	1.26	0.462	0.63	0.234	1.43	0.359
Chromatin structure and dynamics							
2242	Histone deacetylase 1	0.86	0.076	0.97	0.04	0.71	0.05
Transport, transduction and migration							
Signal transduction							
1693	Twitchin	1.7	0.431	1.04	0.302	1.48	0.066
2098	Tyrosine-protein kinase STK	0.67	0.088	0.97	0.028	0.77	0.175
3513	Allograft inflammatory factor 1-like	0.98	0.213	0.7	0.154	1	0.171
4913	Hsc70-interacting protein	1.31	0.227	0.94	0.255	1.52	0.228
5382	Protein kinase C delta type	1.24	0.078	1.05	0.162	1.63	0.174
7045	Calmodulin	1.11	0.328	0.73	0.184	1.5	0.679
Inorganic ion transport and metabolism							
755	Arachidonate 15-lipoxygenase B	0.74	0.27	0.78	0.32	0.55	0.246
Immune and defense response							
Defense mechanisms							
7647	2-iminobutanoate/2-iminopropanoate deaminase	0.66	0.088	0.84	0.065	0.84	0.103
General function prediction only							
190	Complement component C3	1.34	0.108	1.15	0.036	1.7	0.291
3358	Methyltransferase-like protein 27	0.53	0.213	0.9	0.317	0.8	0.341
3382	Methyltransferase-like protein 27	1.47	0.285	1.01	0.144	1.7	0.637
3467	Methyltransferase-like protein 27	0.71	0.057	1.02	0.055	0.62	0.032
3697	Histone-binding protein	0.78	0.04	0.94	0.067	0.72	0.039
3813	Tankyrase-like protein	0.88	0.134	0.97	0.122	0.61	0.093
4508	Inter-alpha-trypsin inhibitor heavy chain H5	0.89	0.342	0.76	0.266	0.73	0.2
4701	Carbohydrate sulfotransferase 15	0.51	0.111	1.14	0.253	0.36	0.09
5645	Methyltransferase-like protein 27	0.79	0.049	0.98	0.023	0.68	0.13
7656	Sodium- and chloride-dependent GABA transporter 2	0.91	0.176	1.91	0.59	1.91	0.539
7893	Pregnancy zone protein	2.39	1.276	1.28	0.183	1.94	0.261
8081	contactin-4-like	0.59	0.19	0.63	0.192	0.42	0.098
Others							

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Table 1 (*continued*)

		HT_117/NC_119		LO_121/NC_119		HL_118/NC_119	
No.	Protein description	Mean ratio	SD	Mean ratio	SD	Mean ratio	SD
308	Beta-catenin-like protein 1	0.76	0.116	0.95	0.14	0.65	0.111
340	Transcription elongation regulator 1	0.75	0.025	1.02	0.104	0.66	0.098
494	Perilipin-2	1.14	0.22	1.09	0.06	2	0.318
665	Forkhead box protein K2	0.85	0.087	0.94	0.178	0.64	0.134
719	60S ribosomal protein L28	1.63	0.284	1.1	0.184	2.14	0.332
754	Tenascin-R	1.24	0.228	1.76	0.4	1.08	0.634
775	Myophilin	1.22	0.34	0.8	0.204	1.55	0.203
819	Charged multivesicular body protein 1a	1.18	0.147	1.06	0.181	1.6	0.036
901	Early endosome antigen 1	1.01	0.164	1.03	0.243	1.59	0.484
919	40S ribosomal protein S21	1.63	0.073	1.12	0.301	1.67	0.35
1017	Copper transport protein ATOX1	1.22	0.135	0.68	0.105	1.39	0.205
1058	60S ribosomal protein L38	1.48	0.335	1.02	0.168	1.78	0.108
1277	Histone H2A.V (Fragment)	1.24	0.34	1.14	0.257	1.66	0.125
1288	Integrin beta	1.33	0.231	1.68	0.348	1.09	0.143
1412	Nucleoplasmin-like protein ANO39	0.89	0.367	0.62	0.029	0.59	0.05
1543	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	0.86	0.14	0.87	0.071	0.67	0.136
2014	Tubulin-specific chaperone A	1.58	0.736	0.92	0.359	3.11	1.776
2077	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	1.11	0.204	0.62	0.119	1.09	0.334
2115	Nuclear receptor subfamily 2 group F member 1-A	0.81	0.153	1.01	0.198	0.57	0.103
2425	Alpha-crystallin B chain	1.46	0.161	1.12	0.122	2.07	0.274
2593	60S ribosomal protein L13	1.74	0.477	1.11	0.148	2.16	0.364
2768	Pre-mRNA-processing-splicing factor 8	0.79	0.057	1.05	0.076	0.65	0.096
2795	Neogenin	0.95	0.097	1.23	0.258	0.62	0.147
2824	Intron-binding protein aquarius	0.84	0.082	0.94	0.083	0.71	0.04
2874	Cohesin subunit SA-1	0.8	0.023	0.93	0.06	0.71	0.049
2907	Heterogeneous nuclear ribonucleoprotein U-like protein 1	0.77	0.004	0.85	0.057	0.65	0.094
2967	Fibrinogen-like protein A	1.03	0.406	1.23	0.147	0.73	0.11
3008	Histone H1-beta, late embryonic	1.3	0.32	1.09	0.341	1.52	0.158
3017	Calumenin-B	1.38	0.341	0.89	0.04	1.53	0.31
3032	Prominin-1-A	2.29	1.926	1.85	0.854	2.02	1.79
3036	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	0.84	0.213	0.81	0.093	0.56	0.18
3062	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	0.75	0.092	0.84	0.058	0.58	0.177
3128	Tumor protein D52	1.52	0.291	1.05	0.116	2.39	0.766
3132	Transient receptor potential cation channel trpm	0.92	0.031	0.96	0.114	0.74	0.002
3165	Semaphorin-1A	2.37	0.887	1.43	0.403	1.41	1.138
3169	Estrogen-related receptor gamma	0.92	0.106	0.99	0.135	0.74	0.106
3319	Ubiquitin carboxyl-terminal hydrolase isozyme L3	1.19	0.263	0.68	0.087	1.12	0.076
3354	Dystonin	0.77	0.127	0.88	0.159	0.54	0.139
3363	Reelin	0.99	0.796	1.35	1.353	0.53	0.321
3493	Protein ELYS	0.86	0.072	1	0.177	0.7	0.025
3796	Sushi repeat-containing protein SRPX	1.31	0.279	1.39	0.154	1.78	0.183
3814	CUB and sushi domain-containing protein 3	0.89	0.083	1	0.234	0.72	0.148
4093	E3 ubiquitin-protein ligase TRIM56	1.36	0.148	1.08	0.297	1.85	0.202
4120	60S ribosomal protein L27	1.57	0.305	1.09	0.211	1.67	0.173
4124	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	0.8	0.036	1.01	0.144	0.66	0.109
4154	U1 small nuclear ribonucleoprotein A	0.88	0.009	0.92	0.064	0.72	0.034
4171	Prelamin-A/C	0.74	0.074	0.98	0.14	0.53	0.144
4295	Methyl-CpG-binding protein 2	0.77	0.043	0.96	0.06	0.68	0.034
4535	40S ribosomal protein S25	1.68	0.608	1.01	0.181	1.88	0.275
4554	Fibrinogen-like protein A	0.83	0.118	0.76	0.056	0.49	0.101
4667	Uncharacterized protein	3.32	0.374	2.37	0.869	1.77	0.244
4773	Crk-like protein	1.34	0.168	1	0.231	1.98	0.409
4840	Ubiquitin-like protein 5	0.99	0.06	0.72	0.064	0.85	0.186
4963	Coiled-coil domain-containing protein 134	1.63	0.365	1.44	0.237	1.66	0.261
5104	Dystonin	0.74	0.111	0.83	0.15	0.55	0.229
5138	Cysteine-rich secretory protein LCCL domain-containing 2	0.76	0.186	0.98	0.189	0.68	0.127
5153	Microtubule-actin cross-linking factor 1	0.86	0.077	0.94	0.018	0.67	0.101
5212	Short-chain collagen C4 (Fragment)	1.12	0.337	1.6	0.155	1.88	0.736
5216	Sorting nexin-5	1.22	0.159	1	0.044	1.58	0.185
5240	Splicing factor 3B subunit 1	0.81	0.063	1.07	0.093	0.72	0.069
5275	THO complex subunit 2	0.85	0.082	1.02	0.029	0.74	0.035
5920	Cleavage and polyadenylation specificity factor subunit 5	0.83	0.118	1	0.096	0.72	0.119
6029	Unconventional myosin-XVIIIa	1.22	0.2	1.05	0.066	1.55	0.235
6096	Serine/threonine-protein kinase PAK 2	1.18	0.241	1.01	0.182	1.62	0.314
6260	Inter-alpha-trypsin inhibitor heavy chain H3	0.88	0.374	0.68	0.254	0.7	0.441
6329	Nucleoprotein TPR	0.83	0.073	0.9	0.054	0.74	0.076
6341	Microtubule-actin cross-linking factor 1	0.9	0.131	0.91	0.093	0.72	0.107
6416	Major yolk protein	1.78	1.047	1.31	0.299	1.1	0.913
6426	Small nuclear ribonucleoprotein Sm D3	0.85	0.113	1.08	0.119	0.73	0.057
6472	Histone H1-beta, late embryonic	1.55	0.466	1.13	0.406	2.02	0.153
6535	Lysozyme 3	2.36	0.514	1.07	0.229	1.8	0.572
6581	Bactericidal permeability-increasing protein (Fragment)	0.72	0.067	0.85	0.036	0.68	0.068
6642	Lipopolysaccharide-binding protein	0.64	0.165	0.68	0.061	0.7	0.131

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Table 1 (*continued*)

		HT_117/NC_119		LO_121/NC_119		HL_118/NC_119	
No.	Protein description	Mean ratio	SD	Mean ratio	SD	Mean ratio	SD
6665	RNA polymerase II-associated factor 1 homolog	0.84	0.093	0.93	0.037	0.65	0.098
6772	Fibrinogen-like protein A	0.79	0.148	1.35	0.327	0.73	0.064
6811	Short-chain collagen C4 (Fragment)	1.57	0.282	1.32	0.273	1.73	0.643
6887	Titin	5	4.454	1.56	1.027	5.47	4.002
7037	Heterogeneous nuclear ribonucleoprotein L	0.83	0.013	1.05	0.046	0.67	0.059
7049	Kinectin	1.24	0.509	1.06	0.273	2.4	0.68
7114	Major yolk protein	1.53	0.773	1.32	0.312	1.08	0.676
7127	Fibrinogen-like protein A	1.82	0.246	1.25	0.592	0.98	0.065
7222	Calbindin-32	0.98	0.204	0.65	0.133	0.86	0.186
7381	Histone deacetylase complex subunit SAP18	0.89	0.076	1.07	0.11	0.73	0.145
7569	Tubulin beta chain	1.1	0.211	1.4	0.66	1.6	0.216
7570	Homeobox protein Hox-A10	0.81	0.093	0.91	0.166	0.6	0.06
7571	Dystonin	0.78	0.113	0.89	0.084	0.59	0.135
7677	Hematopoietic lineage cell-specific protein	1.4	0.454	1.1	0.161	1.76	0.382
7715	40S ribosomal protein S7	1.51	0.257	1.12	0.222	1.67	0.275
7734	Echinoidin	0.7	0.128	0.86	0.16	0.72	0.339
7869	5′-3′ exoribonuclease 2	0.66	0.089	1	0.079	0.54	0.064
7943	Fibrinogen-like protein A	0.97	0.251	1.25	0.683	0.66	0.179
8052	Splicing factor 3A subunit 1	0.86	0.114	1.03	0.01	0.7	0.078
8076	Glutamate receptor 3	0.92	0.183	1.53	0.192	1.33	0.511
8145	Myosin-10	1.09	0.211	0.78	0.25	0.68	0.192
8154	Pre-mRNA-processing factor 40 homolog A	0.84	0.079	1.05	0.082	0.69	0.036
8210	CDGSH iron-sulfur domain-containing protein 2 homolog A	1.49	0.405	1.34	0.289	1.93	0.493
8280	Coactosin-like protein	0.74	0.082	0.82	0.098	0.66	0.095
8286	Myosin-10	0.55	0.294	0.86	0.082	1.28	0.469
8311	Proteasome activator complex subunit 4	0.74	0.021	1.04	0.1	0.62	0.038
8422	Ribosome biogenesis protein BRX1 homolog	0.71	0.11	1.13	0.262	0.6	0.048
8425	KH domain-containing, RNA-binding, signal transduction-associated protein 2	0.86	0.068	1.03	0.116	0.7	0.111
8430	Rho-associated protein kinase 2	1.23	0.207	1.01	0.227	1.6	0.434

significantly enriched in HL group, such as “heterocyclic compound binding” and “organic cyclic compound binding”; Contractile-protein-binding-related GO terms were significantly enriched in LO group, such as “myosin heavy chain binding” and “actin monomer binding”.

From the results of CC categories based on the upregulated proteins in treatment group compared with normal control, ribosome-related GO terms were significantly enriched in HT and HL group, such as “ribosome”, “cytosolic ribosome” and “ribosomal subunit”; Proteins involved in the “extracellular region”, “neuron projection membrane” and “ribosome” were notably represented in the LO group. Based on the

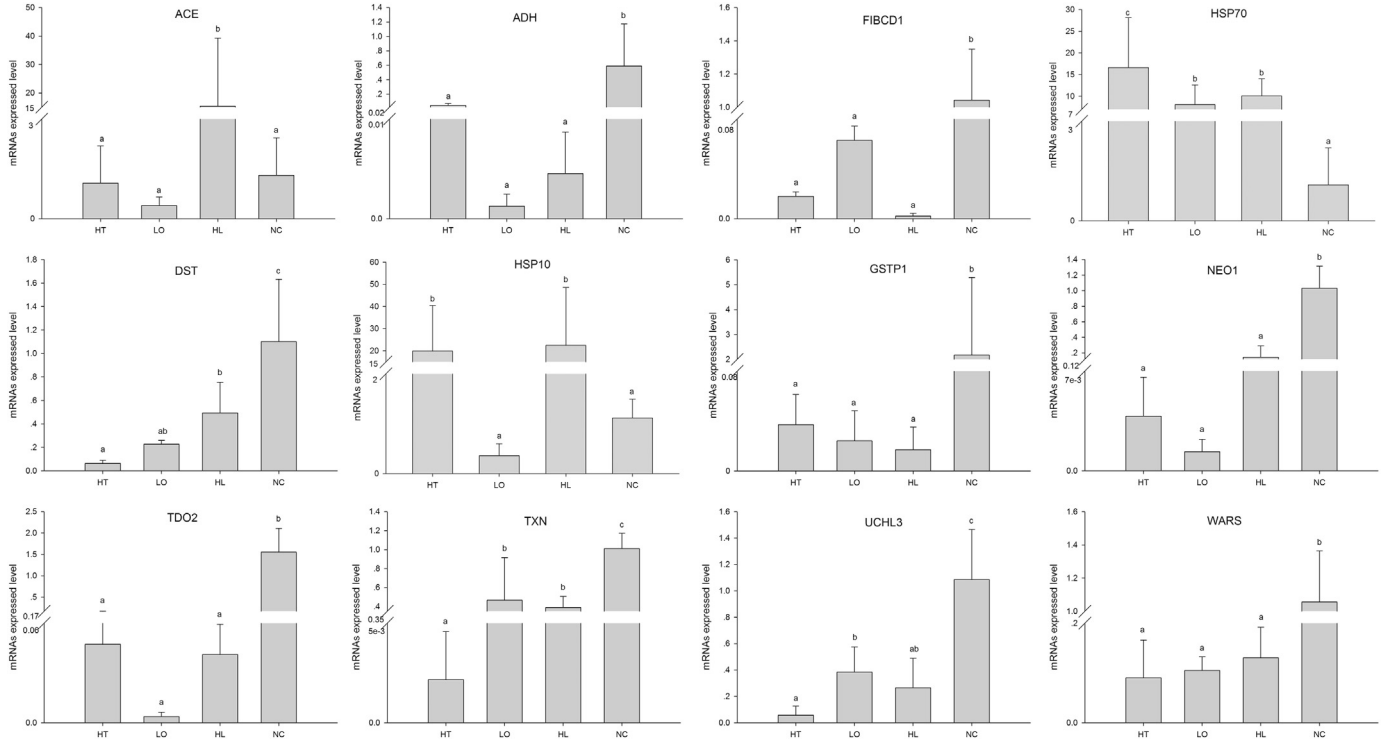

Fig. 3. Real-time PCR analyses for selected genes. Means not sharing a given letter (a and b) differ significantly ($P < .05$). Values indicate the means ± standard deviation ($n = 3$). The definitions of gene abbreviations can be found in Table S1.

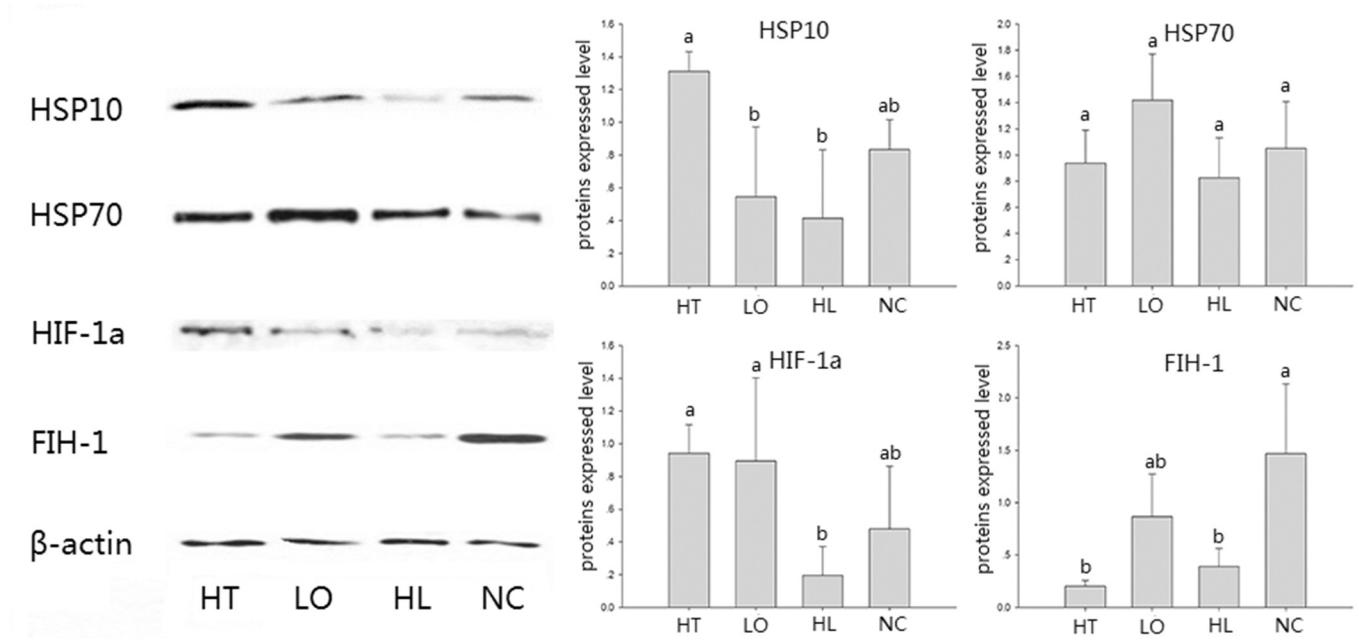

Fig. 4. Western blot analysis of sea cucumber under four treatments. The bands of HSP10, HSP70, HIF-1a, FIH-1 and β-actin from *A. japonicus* illustrate molecular weights of approximately 13, 70, 93, 40 and 42 kDa, respectively. The expression levels of these selected proteins are shown as bars. Means not sharing a given letter (a and b) represented statistically significant differences ($P < .05$).

a
Carbohydrate transport and metabolism 12 9 4
Lipid transport and metabolism 13 3 3
Small molecule transport and metabolism 15 11 3
Energy production and conversion 15 10 5
Secondary metabolites transport and metabolism 16 6 5
Amino acid transport and metabolism 21 16 6
Signal transduction mechanisms 24 12 9
Cell growth and division 24 9 8
General function prediction only 57 28 12
0 20 40 60 80 100 120
Biosynthesis and modification 161 107 25
Others/function unknown 193 100 79
0 50 100 150 200 250 300 350 400
HL_NC HT_NC LO_NC
b
HL_NC
Energy production and conversion 2.72%
Cell growth and division 4.36%
Signal transduction mechanisms 4.36%
General function 10.34%
Biosynthesis and modification 29.22%
Others/function unknown 35.03%
Amino acid metabolism 3.81%
Carbohydrate metabolism 2.18%
Lipid metabolism 2.36%
Small molecule metabolism 2.72%
Secondary metabolites metabolism 2.90%
c
HT_NC
Energy production and conversion 3.22%
Cell growth and division 2.89%
Signal transduction mechanisms 3.86%
General function 9.00%
Biosynthesis and modification 34.41%
Others/function unknown 32.15%
Amino acid metabolism 5.14%
Carbohydrate metabolism 2.89%
Lipid metabolism 0.96%
Small molecule metabolism 3.54%
Secondary metabolites metabolism 1.93%
d
LO_NC
Energy production and conversion 3.14%
Cell growth and division 5.03%
Signal transduction mechanisms 5.66%
General function 7.55%
Biosynthesis and modification 15.72%
Others/function unknown 49.69%
Amino acid metabolism 3.77%
Carbohydrate metabolism 2.52%
Lipid metabolism 1.89%
Small molecule metabolism 1.89%
Secondary metabolites metabolism 3.14%

Fig. 5. The functional analyses of all the differentially regulated proteins identified under environmental stress. (a. total number of differentially regulated proteins in varied biological process under three treatments; proportion of each biological process responded in sea cucumber under b. heat plus hypoxia stress; c. heat stress; d. hypoxic stress;)

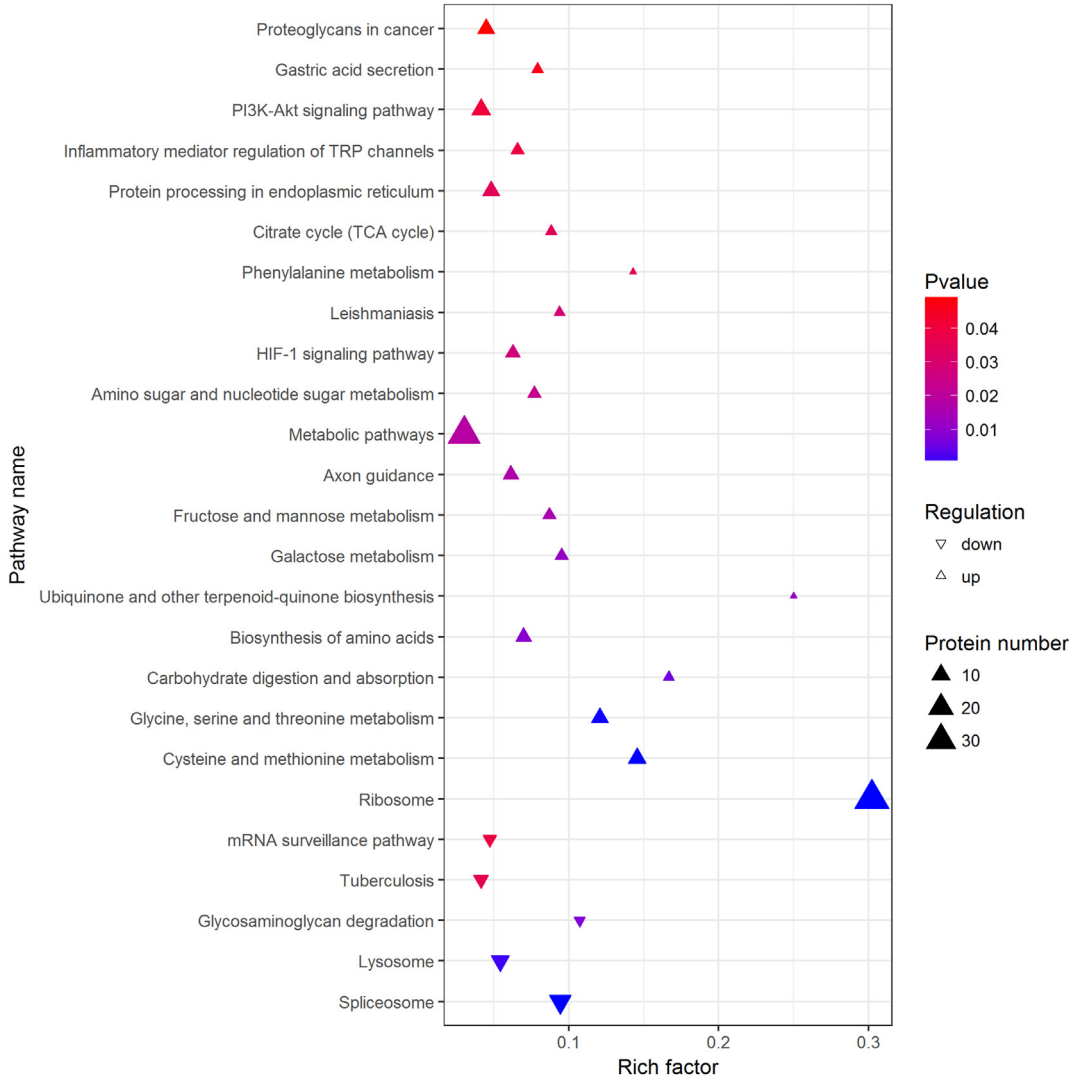

Fig. 6. KEGG pathways analysis of differentially regulated proteins in sea cucumber under heat stress (▲, up-regulated proteins; ▼, down-regulated proteins).

down-regulated proteins in treatment group compared with normal control, nuclear-related and spliceosomal-related GO terms were significantly enriched in HT and HL group, such as “nuclear part”, “nuclear lumen” and “spliceosomal complex”; Moreover, cytoskeleton-related GO terms were significantly enriched in LO group, such as “myofibril”, “myosin complex” and “actin cytoskeleton”. The representative categories in the HL group shared a similarity with the HT group. Thus, we speculated that sea cucumber may adopt a similar strategy to cope with heat stress and heat plus hypoxia stress.

3.4.2. KEGG pathways analyses of up- and down-regulated proteins in sea cucumber under environmental stress

A KEGG pathway enrichment analysis of differentially regulated protein in sea cucumber under heat, hypoxia and the combined stress are shown in Fig. 6, Fig. 7 and Fig. 8, respectively. Compared with the normal control conditions, the up-regulated proteins were significantly enriched in 20, 13 and 39 pathways and the down-regulated proteins were significantly enriched in 5, 21 and 9 in the HT, LO and HL group, respectively ($P < .05$). From the aspect of the common pathways, down-regulated proteins were observed in spliceosome and lysosome pathway in the HT and HL group; the up-regulated proteins were significantly enriched in ribosome pathway in all the three groups. The specific pathways in each group could be found in the figures.

3.4.3. Key responses in sea cucumber to environmental change

Based on COG, GO and KEGG analyses, changes in proteins functioning in “substance biosynthesis, transport and metabolism”, “signal transduction”, “synthesis and modification”, “immune and defense response”, and “energy production and conversion” are key responses in sea cucumber to environmental change. The effect of different climate change stressors, individually and in combination, on the respiratory tree in sea cucumber as indicated by the differential expression of proteins and associated metabolic pathways are summarized in Fig. 9. The results of key responses might suggest that a different adaptation strategy is developed by sea cucumber in response to varied environmental stress.

Take the category of “substance biosynthesis, transport and metabolism” as an example, significantly regulated proteins involved in amino acid metabolism related pathways were up-regulated in the HT and HL group, such as “tyrosine metabolism” and “alanine, aspartate and glutamate metabolism” (Figs. 6 and 8). Proteins, which enriched in amino-acid-metabolism-related GO categories (sulfur amino acid, cellular amino acid and alpha-amino acid metabolic process) were mainly down-regulated in the LO group but up-regulated in the HT group in sea cucumber. Moreover, significantly regulated proteins involved in carbohydrate metabolism, such as carbohydrate metabolic and biosynthetic process, were mainly up-regulated in the HT group but showed conversely trends in the LO group. Increased expressions of proteins

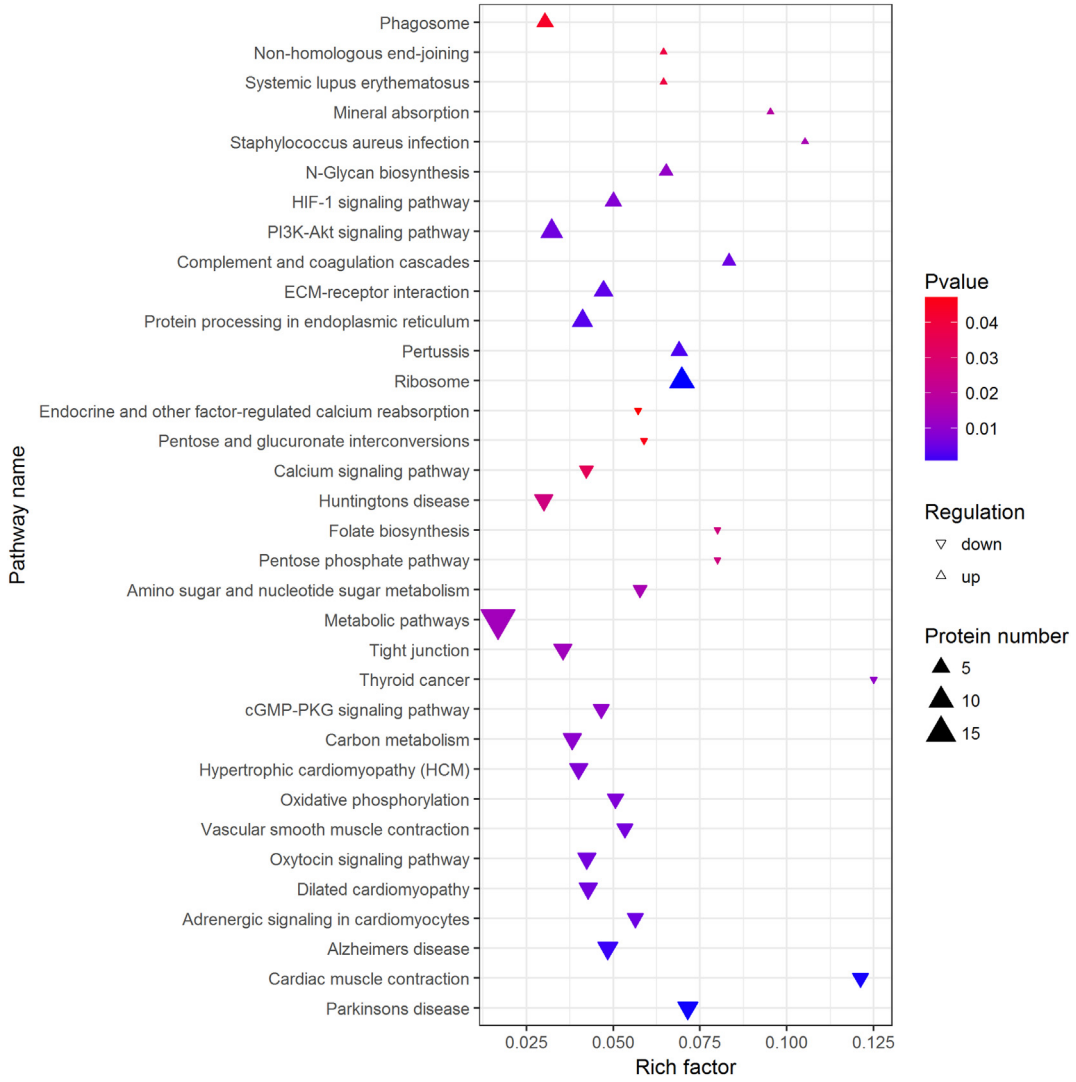

Fig. 7. KEGG pathways analysis of differentially regulated proteins in sea cucumber under hypoxia stress (▲, up-regulated proteins; ▼, down-regulated proteins).

involved in "fructose and mannose metabolism" and "galactose metabolism" indicated that carbohydrate metabolism was up-regulated under heat and heat plus hypoxia stress. What is more, in the current study, proteins involved in (cellular) lipid metabolic process and negative regulation of proteolysis were significantly down-regulated in sea cucumber under heat with or without hypoxia. However, an up-regulation in GO categories of (cellular) lipid catabolic process, lipid oxidation and modification were observed in HL group. KEGG pathway analysis showed that lipid metabolism related pathways (arachidonic acid and sphingolipid metabolism) were down-regulated in HL group and pathways related with lipolysis (fatty acid elongation and degradation) were up-regulated. In conclusion, together with the results of the amino acid and carbohydrate changes under different environmental stress, we hypothesize that metabolic pathway alterations were dependent on the type of stressor.

4. Discussion

Although the recent focus of research on climate change influence has been toward aquatic animals, there is still lacking the study about the combined effects caused by multiple stressors. In the current study, a total of 8437 proteins were identified in protein-expression profiles of sea cucumbers respiratory tree under single and multiple environmental stresses. Differential proteomic analyses of *A. japonicus* under heat with or without hypoxia were performed, and the findings are further discussed below. These proteomic changes in response to high-temperature and low-oxygen levels may provide insights into the defense strategies of sea cucumber in response to global climate changes including heat and hypoxia.

4.1. Signal transduction

4.1.1. Key signaling pathways

As reported previously, alterations of $[Ca^{2+}]_i$ represent a first-level response of many cell types to hypoxia, and could affect the modulation of signaling pathways [57]. Another previous study suggested that the downregulation of PKG protein and/or activity might because of the generation of ROS and RNS during hypoxia [58], and it was also demonstrated in fruit fly (*Drosophila melanogaster*) that the cGMP–PKG–PP2A pathway could alter behavioral tolerance to acute hypoxia [59]. Thus, the significantly down-regulated signal-transduction-related pathways, like cGMP-PKG signaling pathway and calcium signaling pathway, could be explained in the current study. Similar down-regulation in oyster (*Crassostrea gigas*) under multiple climate change stressors has also been reported [33]. Besides, PI3K/Akt pathway could stimulate proliferation and survival [60], and activated PI3K could play a role in the inhibition of cells apoptosis and the stabilization of HIF-1a [61], which is a transcription factor that regulates

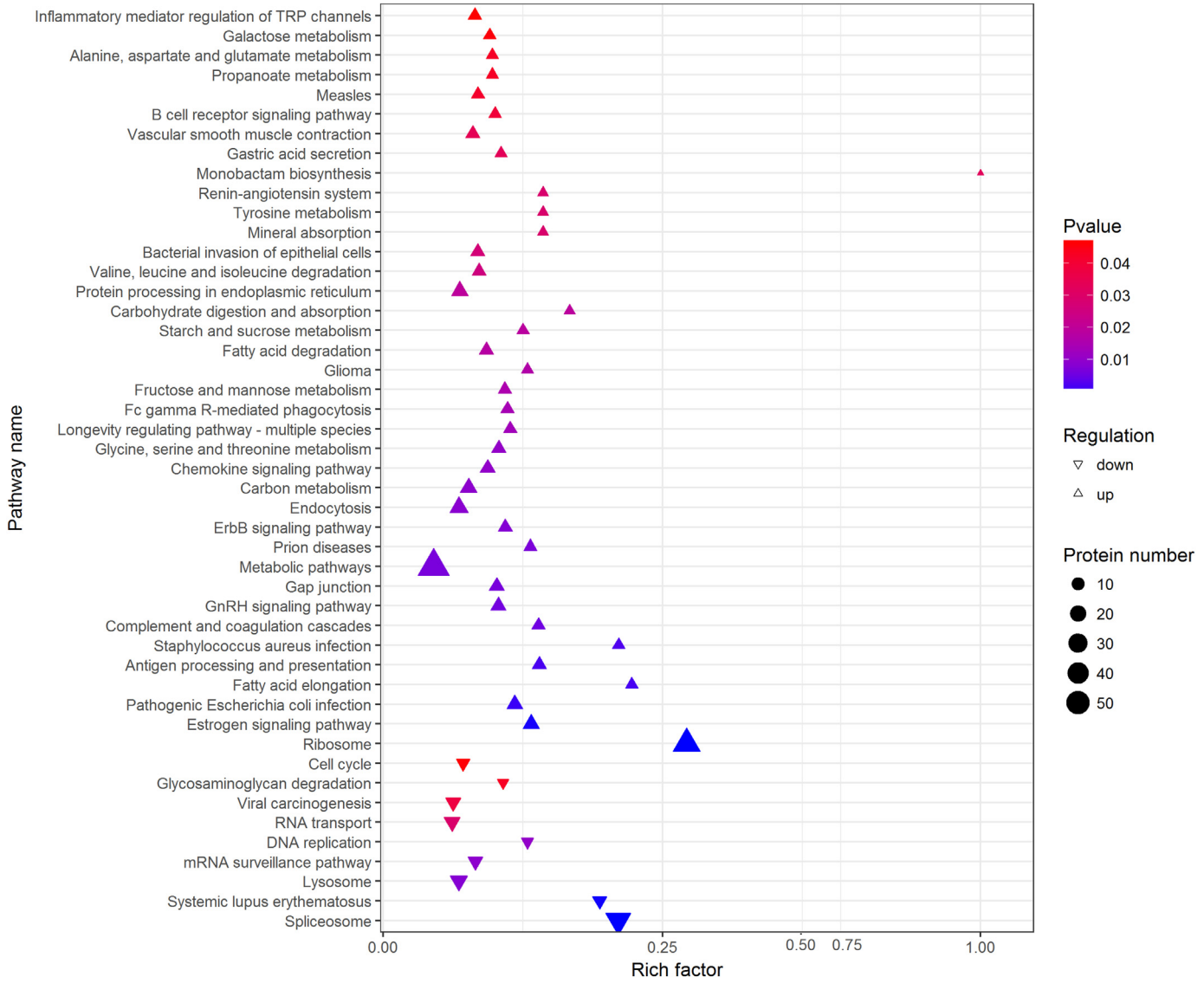

Fig. 8. KEGG pathways analysis of differentially regulated proteins in sea cucumber under heat plus hypoxia stress (▲, up-regulated proteins; ▼, down-regulated proteins).

oxygen homeostasis and could be induced in response to hypoxia, by reducing expression of heat shock proteins [62,63]. Therefore, significantly up-regulated proteins involved in HIF-1 signaling pathway and PI3K-Akt signaling pathway might be essential for the survival and adaptation of sea cucumber to a stressed environment.

4.1.2. Iron homeostasis

Iron is related with oxygen homeostasis, because of its essential role in incorporating into the heme group, which is a key element of both hemoglobin and myoglobin [64,65]. In the current study, significantly up-regulated proteins enriched in iron ion binding and homeostasis related GO terms were observed, including toposome, major yolk protein (MYP) and major yolk protein 2. MYP contains transferrin-like iron binding domains, and it was supposed to bind and transport iron in sea urchins (*Strongylocentrotus purpuratus* and *Lytechinus variegatus*) [66]. Moreover, ferritin is a key protein involved in iron storage that might defend bacterial infection by regulating free iron in the environment [41,67]. In the present study, up-regulation of ferritin in sea cucumber under heat plus hypoxia stress, was consistent with the earlier report on grass shrimp *Palaemonetes pugio*, where the expression of *ferritin* gene was up-regulated following chronic hypoxia exposure [68]. Thus, iron ion was supposed to be responsive in signal transduction of sea cucumber under environmental stress, especially when exposed to hypoxia, even though the exact mechanism needs further elucidation.

4.2. Protein biosynthesis

The general notion is that hypoxia would reduce the overall level of protein synthesis [69], and it has been reported in grass carp (*Ctenopharyngodon idella*) and broccoli sprouts (*Brassica oleracea* var. *italica*) and in the sea cucumber *A. japonicus* at transcriptional level [70–72]. Situation was quite similar in incubating cells under elevated temperatures [73]. Peptide methionine sulfoxide reductase (MsrA) could mediate the reduction of Met(O) to Met, both as a free amino acid and when incorporated into proteins and it was reported to play an important role in protecting cells against oxidative damage [74,75]. Moreover, ELAV-like protein 2, which is involved in post-transcriptional regulation of mRNA turnover and mRNA stability [76]; WARS participates in the first step of protein synthesis by invoking tryptophan to form Trp-AMP [77]. In the current study, these proteins were significantly down-regulated under environmental stress, thus leading to the speculation about the depressed ability of protein synthesis in the

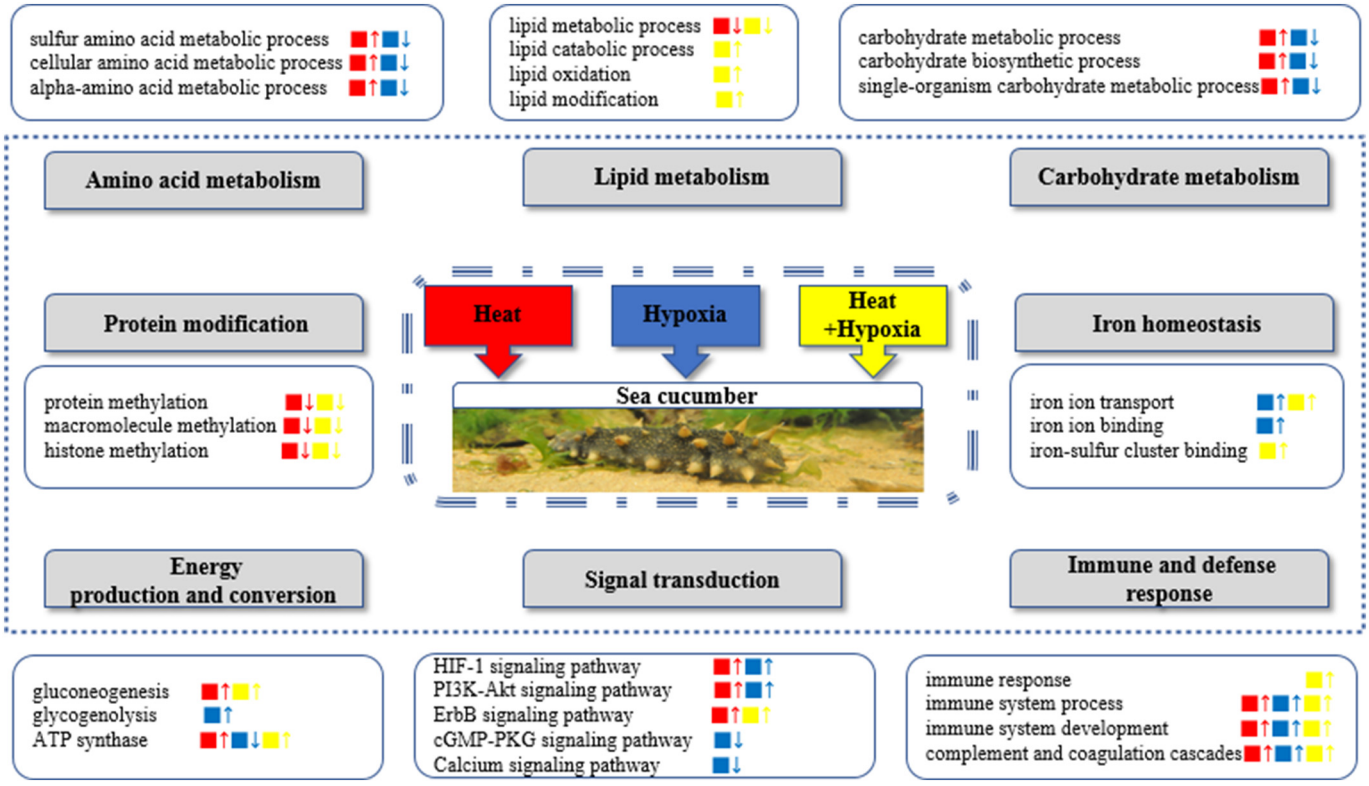

Fig. 9. A schematic model of the response mechanism in the respiratory tree of *A. japonicus* under environmental stress (Responded changes to heat, hypoxia, and heat plus hypoxia are shown in red, blue and yellow, respectively. Up and down arrows represent the up-regulated and down regulated process, respectively.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sea cucumber in adverse environments. A potential reason might be that reducing protein synthesis could avoid exposing nascent polypeptides to denaturing conditions and thus allow for a notable cellular energy saving to cope with undesirable environments [10,78].

4.3. Immune and defense response

Changed immune activity has been observed in many marine organisms exposed to environmental stress. For instance, hypoxic stress could increase the abundance of immune-related proteins in the Chinese shrimp (*Fenneropenaeus chinensis*) [41]. Previous studies about *A.japonicus* showed that immune enzyme activities were significantly altered under acute temperature and DO content changes [19,49]. As is commonly believed that non-specific immune response (innate response) is the type of immune response in sea cucumber [79]. The complement system is essential for the innate defense against common pathogens and could be activated by oxidative stress [80,81]. Complement component C3 is an intermediary between the innate and adaptive immune system and is thought to provide protection against oxidative stress [80,82]. The significantly up-regulated C3 expression in the present study was also in line with the previous study about Iberian ribbed newt (*Pleurodeles waltl*) under heat shock [82]. Therefore, we speculated that environmental stress could induce C3 expression, thus leading to the activation of the immune system.

Heat shock proteins (HSPs) were illustrated to play an important role in survival and develop thermotolerance by participating in innate and adaptive immune responses and impair cellular damage related with stresses [83,84]. As a bioindicator of thermal stress in the sea cucumber [85], HSP70 was also demonstrated to be induced by hypoxia stress in several aquatic organisms [49,86–88]. HSP10, another heat shock protein, is important in the inhibition of immunoinflammatory reaction and was also found downregulated in Indian Catfish *Clarias batrachus* under hypoxia [89] [90]. HSP10 is seemed as an antagonist of other extracellular heat shock proteins and the correlation between decreased level of HSP10 and upregulated immune response was supposed [90]. Thus, the expression patterns about HSP70 and HSP10 in the present study could be partially explained. HSPs play important roles in enhancing survival upon environmental stress by participate in immune process and potential more processes. The relation between more HSPs expression and stress are expected to be elucidated by future studies.

4.4. Energy production and conversion

Under hypoxia, anaerobic metabolism is the major approach for obtaining energy in marine organisms [43]. Glucose-6-phosphate isomerase (GPI) is an essential enzyme involved in glycolysis and gluconeogenesis [91] and could catalyze the reversible interconversion of fructose-6-phosphate and glucose-6-phosphate [92]; NADH dehydrogenase and cytochrome *c* oxidase subunit 5A are involved in electron transport chain [93]. The depression of these proteins under hypoxia stress indicates that glycogen synthesis is suppressed and glycogenolysis is enhanced. This result was in line with the conclusion in rainbow trout (*Salmo gairdneri*) during environmental hypoxia [94]. However, the induced expression of pyruvate carboxylase in the current study, indicating that the synthesis of phosphoenolpyruvate from pyruvate during gluconeogenesis was prompted under heat stress [95]. Moreover, phosphoenolpyruvate carboxykinase, which plays a rate-limiting role in gluconeogenesis, and the up-regulated expression indicates that gluconeogenesis was induced in sea cucumber under heat plus hypoxia stress, and this conclusion is consistent with a previous study in lactating Holstein cows and Saluki dog under heat stress [96,97]. Sea cucumber seems to elevate energy production and conversion under adverse environments but takes varied strategies toward gluconeogenesis/glycogenolysis under heat and hypoxia stress. More proteins and genes involved in this process need to be further measured.

Higher temperature was thought to be an indication of higher ATP demand [46], and it mediates the last step of oxidative phosphorylation to regenerate ATP from ADP and Pi [98] Up-regulation of ATP synthase-related proteins in the present study, like ATP-citrate synthase under heat stress and ATP synthase-coupling factor 6 as well as ATP synthase subunit O under heat plus hypoxia stress, suggesting a strategy to compensate the urgent need for energy in sea cucumber under heat stress. Besides, ATP synthase is also involved in aerobic respiration [99]. When there is a deficiency of available DO, aquatic animals would resort to anaerobic respiration to maintain the energy demand rather than aerobic metabolism [100]. Thus, the down-regulated expression of ATP synthase caused by subdued aerobic metabolism under a hypoxic environment was not surprising, and the same is for Pacific Oyster *C. gigas* and Chinese shrimp *F. chinensis* [41,101]. Taken together, these results suggest that sea cucumber can differentially regulate the expression of energy-related proteins as a compensatory response to varied environmental stress.

5. Conclusion

High temperature and low dissolved oxygen are common challenges for aquatic organisms in the context of climate change. However, seldom study has characterized iTRAQ-based quantitative proteomic analysis of echinoderms in response to environmental stress. This study provides a global view of the proteins that are differentially regulated in the respiratory tree of *A. japonicus* under climate change related stressors (heat, hypoxia, and their interaction) using the iTRAQ technique. Significant responsive proteins from *A. japonicus* to both individual and interacting environmental stress, which were involved in diverse biological pathways were identified in this work. Alterations of defense strategies in sea cucumber are revealed to be normally dependent on the type of stressor. These proteome-wide alterations, including “substance biosynthesis, transport and metabolism”, “signal transduction”, “protein synthesis”, “immune and defense response” and “energy production and conversion” reflect an altered strategy that may enable *A. japonicus* to survive under global climate change in the future. More differentially regulated proteins were identified in sea cucumber under stress from heat and hypoxia stress than from a single stress, indicating that the interaction between these two stressors has an additive effect at the proteome level. These results provide novel insights into the proteins involved in the response of *A. japonicus* to heat, hypoxia and the combined stresses and will facilitate further investigation about possible molecular events in sea cucumber under climate change. Our study also provided a possibility for taking sea cucumber as the typical echinoderm species in the research of stress resistance under complicated and flexible aquatic environment.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.12.020. Raw data are available via ProteomeXchange with identifier PXD008472.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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